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14. ABSTRACT

The lacZ gene encoding E. coli beta-gal has already been recognized as the most commonly used reporter system in cancer gene therapy. Moreover, prostate-specific membrane antigen (PSMA) has been identified as an ideal antigenic target in prostate cancer. We propose to develop a novel class of Gd(III)-based MRI contrast agents for in vivo detection of beta-gal or PSMA activity. This new concept of the Gd(III)-based MRI contrast agents is composed of three moieties: (A) a signal enhancement group, such as Gd-DOTA or Gd-PCTA; (B) an Fe(III) chelating group; (C) beta-D-galactose or glutamate. Following cleavage by lacZ transgene or PSMA in prostate cancer cells, the released, activated aglycone Fe(III)-ligand will spontaneously trap endogenous Fe(III) at the site of enzyme activity forming a highly stable complex, to restrict motion of the Gd(III) chelates enhancing relaxivity and providing local contrast accumulation. We plan to synthesize 8 novel MRI contrast agnets for imaging beta-gal or PSMA activity in prostate cancer cell culture, explore the feasibility of applying the most promising analogies to cells grown in vivo in mice and rats.

15. SUBJECT TERMS
Prostate Cancer Evaluation, Contrast Agent, Synthesis, MRI, Gene Expression, Gene Therapy, in vivo Cancer Imaging, lacZ Gene, beta-Galactosidae, PSMA, NAALADase

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INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the leading cause of cancer death in men in the United States, an estimated 218,890 new cases with 27,050 deaths in 2007.[1,2] Gene therapy has emerged as a potentially promising strategy for treatment of prostate cancer.[3-15] The prostate is particularly amenable to gene therapy.[11-16] However, there are major issues in terms of assessing the delivery to target tissue, assessing the uniformity (versus heterogeneity) of biodistribution and determining whether the genes are expressed.[15-33] A viral construct is often readministered on successive occasions, but this should optimally be timed to coincide with loss of expression. Inevitably gene therapy has associated risks, and thus non-invasive *in vivo* determining the duration of gene expression in an individual tumor could greatly enhance the viability of the approach.

Gene expression now is commonly monitored by *in situ* hybridization techniques or by introducing a marker gene to follow the regulation of a gene of interest. Since β-galactosidase (β-gal) activity is readily assessed by histology or in culture, in hosts as evolutionarily diverse as bacteria, yeast, and mammals, its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction, *lacZ* gene encoding *E. coli* β-gal has already been recognized as the most commonly used reporter system.[34] However, the well-established chromogenic or fluorogenic substrates, relying on the hydrolysis by β-gal to release colorful compounds are limited to histology or *in vitro* assays.[35-39] Non-invasive *in vivo* detecting of transgene expression would be of considerable value in many ongoing and future clinical gene therapy trials.

The superb spatial resolution and the outstanding capacity of differentiating soft tissues have determined the widespread success of magnetic resonance imaging (MRI) in clinical diagnosis.[40] The contrast in an MR image is the result of a complex interplay of numerous factors, including the relative T_1 and T_2 relaxation times, proton density of the imaged tissues and instrumental parameters. It was shown that contrast agent causes a dramatic variation of the water proton relaxation rates, thus providing physiological information beyond the impressive anatomical resolution commonly obtained in the uncontrasted images. Contrast agents are widely used clinically to assess organ perfusion, disruption of the blood–brain barrier, occurrence of abnormalities in kidney clearance, and circulation issues.[40-44] The responsive MRI contrast agents holds great promise in the gene therapy arena.[45,46] The abilities of these contrast agents to relax water protons is triggered or enhanced greatly by recognition of a particular biomolecule opening up the possibility of developing MRI tests specific for biomarkers

indicative of particular disease states and aiding in the early detection and diagnosis of tumors. Desreux *et al* [42,47] demonstrated that, by chelating Gd(phen)HDO3A with Fe(II) to form a highly stable tris-complex, as shown in **Figure 1**, the relaxivity increased 145% at 20MHz and 37°C from 5.1mM^{-1}s^{-1} per Gd(III) in Gd(phen)HDO3A form to 12.2 mM^{-1}s^{-1} in the tris-complex. Desreux *et al* [42,47] also synthesized another iron-sensitive MRI contrast agent with a tris-hydroxamate (**Figure 2**). After the tris-hydroxamate groups formed a chelate with Fe(III), free rotation at the Gd(III) centers was restricted, thereby increasing relaxivity by 57% from 5.4 to 8.5mM^{-1}s^{-1} at 20 MHz.

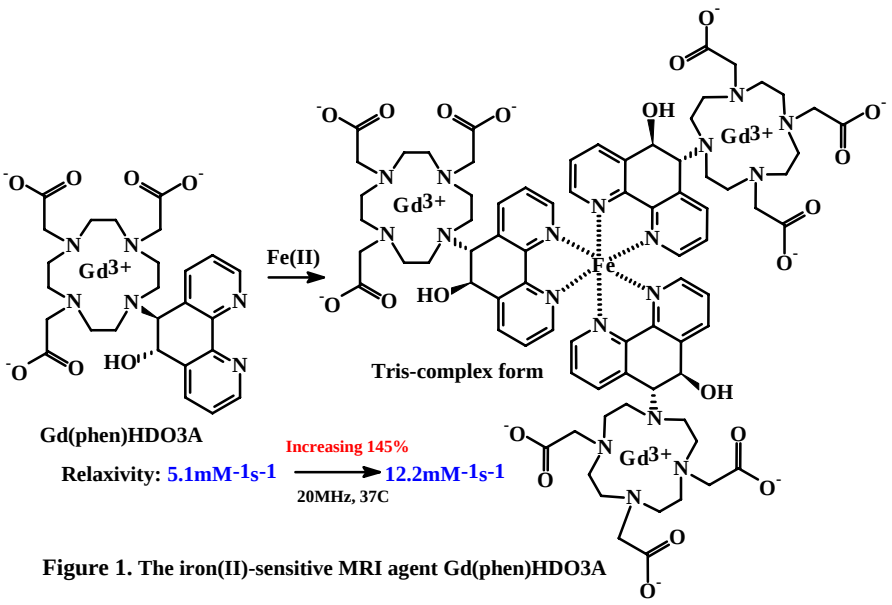

Figure 1. The iron(II)-sensitive MRI agent Gd(phen)HDO3A

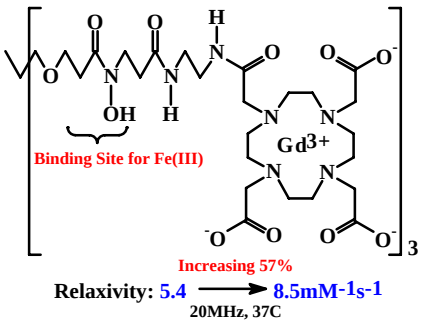

Figure 2. The iron(III)-sensitive MRI agent Gd(III)-Trishydroxamic acid

Iron is a critically important metal ion for a wide variety of cellular events.[48] Tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilization of iron, as evidenced by an increase in transferrin receptors at the cell surface.[49-51] Additionally, cancer cells are sensitive to the effects of iron chelators because of the critical requirement for iron in proteins that play essential roles in DNA synthesis and energy production.[52,53] Such studies have led to iron chelation therapy to clinically treat some tumors.[54-58]

Based on the MRI contrast agents findings and the biologic features of tumor, we have proposed in this project a novel class of enzyme activated Gd^{3+}-based MRI contrast agent for *in vivo* detection of β-gal activity, in which we try to combine all means of reaching the highest possible relaxivities (**Figure 3**).[42,47]

Additionally, prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein with enzymatic activities: N-acetylated α-linked L-amino dipeptidase (NAALADase) and γ-glutamyl

carboxypeptidase (folate hydrolase).[59-61] Studies with the monoclonal antibodies have demonstrated that PSMA is the most well-established, highly restricted prostate cancer cell surface antigen, it is expressed at high density on the cell membrane of all prostate cancers.[62-64] The high prostate tissue specificity of PSMA has been identified as an ideal therapeutic and diagnostic target for prostate cancer, this potential was exemplified by the recent FDA approval of an ^{111}In-labeled PSMA monoclonal antibody (Prostascint®) for diagnostic imaging of prostate cancer.[65-67] Furthermore, phase I and II trials have begun using immunotherapy directed against PSMA.[68-70] By introducing γ-glutamate residue instead of D-galactose in our proposed above new mechanism diagram, we intend to develop a novel class of Gd(III)-based MRI contrast agents for *in vivo* imaging prostate tumor through PSMA activated *in situ* Fe^{3+}-trapped MRI contrast agent formation (**Figure 4**).

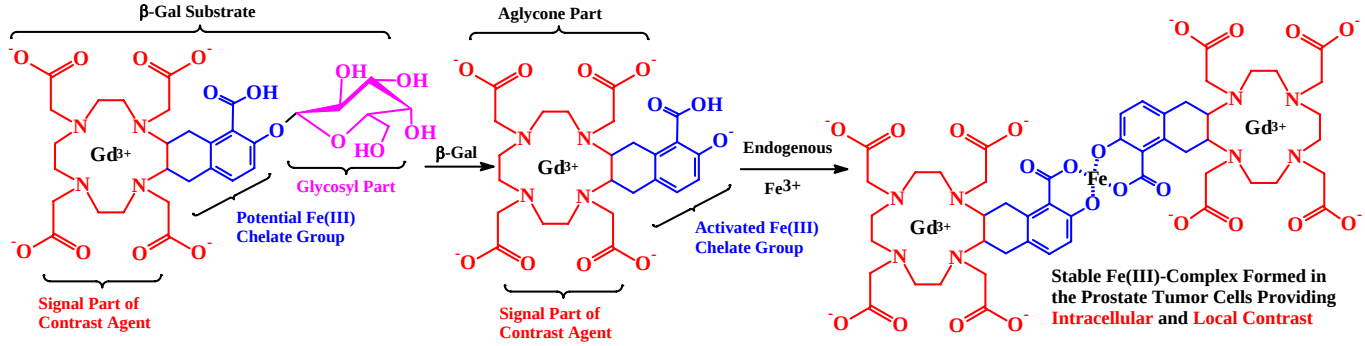

Figure 3. Mechanism of proposed new platform for *in vivo* detection of *lacZ* gene expression through β-gal activated *in situ* Fe^{3+}-trapped MRI contrast agent formation.

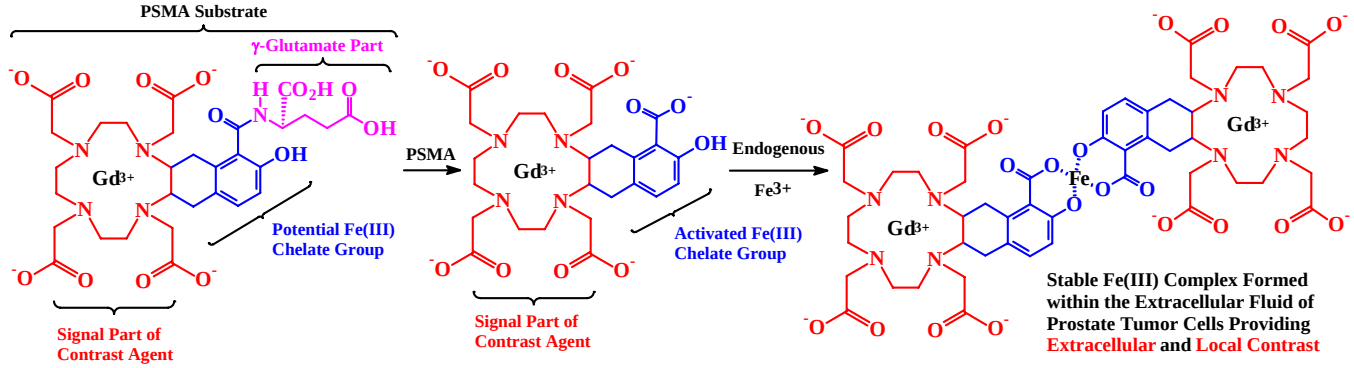

Figure 4. Proposed new mechanism for *in vivo* imaging prostate tumor through PSMA activated *in situ* Fe^{3+}-trapped MRI contrast agent formation.

Especially, PSMA has a large extracellular domain,[70] so the expression of PSMA tethered to the surface of the prostate cancer cells makes that the novel peptide-based MRI contrast agents can be targeted for activation within the extracellular fluid of prostate cancers [71] and overcomes the need for a peptide-based MRI contrast agent to penetrate the tumor cell membrane, thus, providing *in vivo* prostate

cancer imaging through an **extracellular** MRI approach. The concern of permeability is one of the greatest challenges in the development of *in vivo* MRI contrast agents.[72]

Accordingly, depending upon the enzyme sources either being the *lacZ* transgene or the PSMA from prostate tumors, this new platform could provide *in vivo lacZ* gene expression assay or *in vivo* prostate cancer imaging (in particular, through **extracellular** contrast agents), with combining all the approaches of reaching the highest possible relaxivities.[42,47,72] Furthermore, this new class of responsive MRI contrast agent is composed of three functional moieties, in which the signal enhancing and Fe^{3+} chelating parts are flexible allowing modification in a search for ideal Fe^{3+}-trapped MRI contrast agents. Importantly, the combination of three functional moieties is based on the clinically applied strategies on cancer therapy. These facts strongly suggest the potential of the proposal to future clinical application.

Most recently, Merbach *et al* [73-76] observed the remarkably high T_1 relaxivity gain by the heterometallic, self-assembled metallostar formation with six efficiently relaxing Gd^{III} centers from (tpy-DTTA)Gd(H_2O) with 7.3$mM^{-1}s^{-1}$ to $\{Fe^{II}[Gd^{III}_2(tpy\text{-}DTTA)_2(H_2O)_4]_3\}^{4-}$ with 15.7$mM^{-1}s^{-1}$ at 20MHz and 37°C (**Figures 5**), significantly, their detailed studies on structure and dynamics of the trinuclear complex $\{Fe^{II}[Gd^{III}_2(tpy\text{-}DTTA)_2(H_2O)_4]_3\}^{4-}$ indicate that the heterometallic self-assemblies attain high T_1 relaxivities by influencing three factors: water exchange, rotation, and electron relaxation, which are fully consistent with the expecting results shown as above in **Figures 3** and **4**, the effectiveness of contrast agents can be increased by restricting the motion of Gd(III) chelates by linking them rigidly to macromolecules through covalent or non-covalent bonds, by an improvement of their intrinsic relaxivity or by attaching several paramagnetic entities to biological or synthetic oligomers. Obviously, these most recently comprehensive investigations as relevant evidences strongly support for our current proposal.

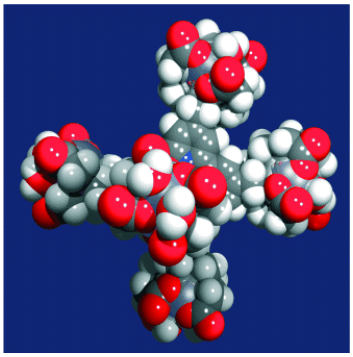

Figure 5. $[Fe\{Gd_2L(H_2O)_4\}_3]^{4-}$

STATEMENT OF WORK

Specific Aim 1 Design and synthesize model "smart" MRI contrast agents to report β-gal or PSMA activities with the ability of trapping Fe^{3+} ion.

Task 1 Design and optimization of synthetic strategies for reporter molecules. (**Months 1-18**)

Task 2 Structural characterizations of the synthesized molecules. (**Months 4-20**)

Specific Aim 2 Test the properties of molecules in solution and *in vitro* with cultured prostate cancer cells.

Task 3 Evaluation the basic properties of the agents **in solution**. (**Months 20-22**)

Task 4 Evaluation of the properties of the optimal molecules *in vitro* **with cultured prostate cancer cells**. (**Months 23-25**)

Specific Aim 3 Scale up synthesis of the most promising MRI contrast agent(s) and apply to animal investigations.

Task 5 Scale up synthesis of the most promising ^{1}H MRI contrast agent(s). (**Months 26-28**)

Task 6 Apply the most promising ^{1}H MRI contrast agent(s) to assess β-gal transfection efficiency, *lacZ* gene expression (spatial and temporal) in prostate tumors *in vivo* (48 mice + 48 rats). (**Months 29-35**)

Task 7 Test dosing protocols, timing, MR detection protocols (48 mice) (**Months 29-35**)

Task 8 Prepare manuscripts and final report (**Month 36**)

BODY

In this no-cost extension year, our work continued followed the research plan of the approved proposal W81XWH-05-1-0593 on: **Task 1** Design and optimization of synthetic strategies for reporter molecules; **Task 2** Structural characterizations of the synthesized molecules; **Task 3** Evaluation the basic properties of the agents in solution; and **Task 4** Evaluation of the properties of the optimal molecules *in vitro* with cultured prostate cancer cells; **Task 5** Scale up synthesis of the most promising ^{1}H MRI contrast agent(s); **Task 6** Apply the most promising ^{1}H MRI contrast agent(s) to assess β-gal transfection efficiency, *lacZ* gene expression (spatial and temporal) in prostate tumors *in vivo*; **Task 7** Test dosing protocols, timing, MR detection protocols (48 mice).

Through three years supported by DOD W81XWH-05-1-0593, we have successfully obtained the target reporters $\mathbf{M_1}$, $\mathbf{M_3}$, $\mathbf{M_5}$, $\mathbf{M_7}$, $\mathbf{M_9}$ and $\mathbf{M_{10}}$ designed in the proposal by the modified synthetic strategies and routes (see **Figure 1**). The MRI evaluation of the reporter molecules $\mathbf{M_1}$, $\mathbf{M_3}$, $\mathbf{M_5}$, $\mathbf{M_7}$, $\mathbf{M_9}$ and $\mathbf{M_{10}}$, respectively, in sodium phosphate buffer solution (PBS) (0.1 M, pH=7.4) in the presence of ferric ammonia citrate (FAC) with β-galactosidase E801A indicated that: (1) the reporter molecules $\mathbf{M_1}$, $\mathbf{M_3}$, $\mathbf{M_5}$, $\mathbf{M_7}$ can not be hydrolyzed by β-galactosidase E801A, so no MRI contrast changes before and after addition of β-galactosidase E801A can be seen; (2) only reporter molecules $\mathbf{M_9}$ and $\mathbf{M_{10}}$ can be hydrolyzed by β-galactosidase E801A in the presence of FAC in PBS (0.1 M, pH=7.4), producing

obvious MRI contrast changes before and after reaction with β-galactosidase E801A (see **Figure 2**), it implies that the released aglycone including the activated Fe^{3+}-ligand and MRI signal enhancement group spontaneously traps Fe^{3+} in the solution forming a highly stable complex, then restricting the motion of the Gd^{3+} chelates enhancing relaxivity.

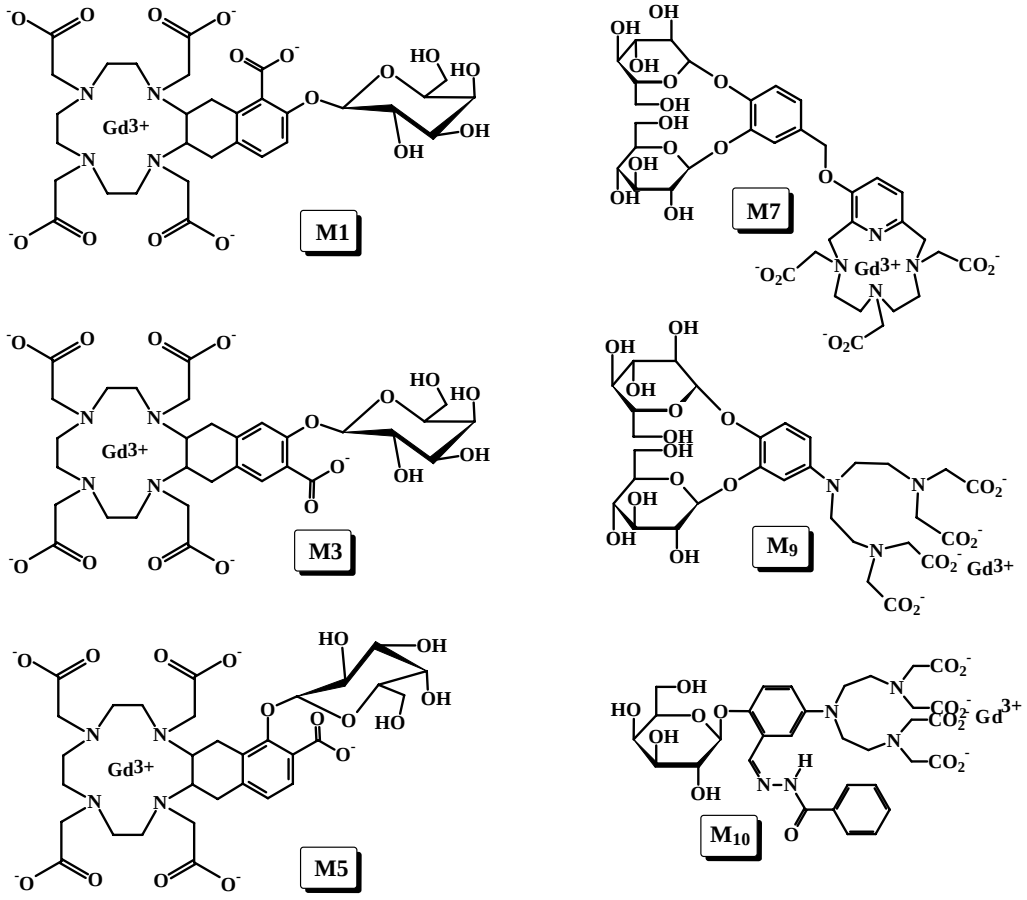

Figure 1. The Structures of M_1, M_3 M_1, M_3 and $M_5$and M_5

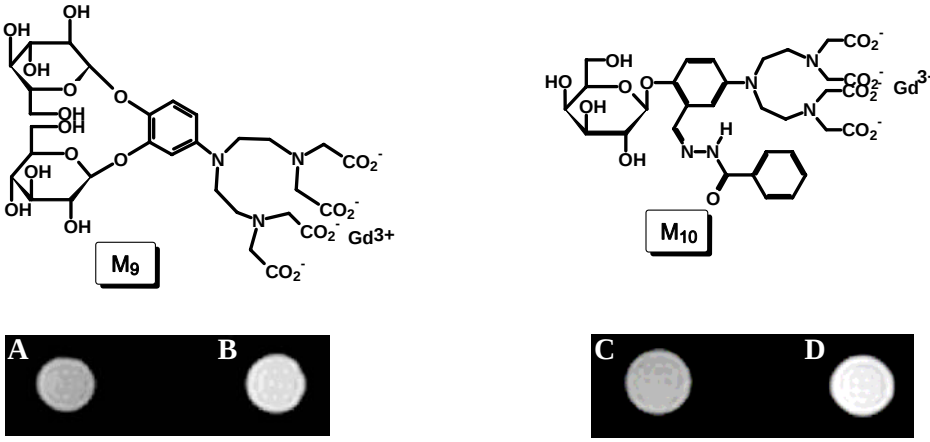

Figure 2. *T*1-weighted (TR/TE 250/12 ms) MR images of solutions and the signal intensity in test tubes at 4.7 T MR scanner: (**A**) PBS with $\mathbf{M_9}$ and FAC; (**B**) PBS with $\mathbf{M_9}$, FAC and β-galactosidase E801A; (**C**) PBS with $\mathbf{M_{10}}$ and FAC; (**D**) PBS with $\mathbf{M_{10}}$, FAC and β-galactosidase E801A.

***In Vitro* MRI Studies of M_9 and M_{10}**

(1) Cell preparation (**a**) Stably transfected PC3 cell line: *E. coli lacZ* gene (from pSV-β-gal vector, Promega, Madison,WI) was inserted into high expression human cytomegalovirus (CMV) immediate-early enhancer/promoter vector phCMV (Gene Therapy Systems, San Diego, CA) giving a recombinant vector phCMV/*lacZ*, which was used to transfect PC3 cells using GenePORTER2 (Gene Therapy Systems). Cells were grown in DMEM (Dulbecco's Modification of Eagle's Medium, Mediatech, Inc, Herndon, VA), 10% FBS (Fetal bovine serum, Hyclone, Logan, UT) with 1% Penicillin-streptomycin Solution (Mediatech). The highest β-gal expressing colony was selected using G-418 disulfate ($C_{20}H_{40}N_4O_{10}$. $2H_2SO_4$, Research Products International Corp, Mt. Prospect, IL) (800 μg/ml), which was also included for routine culture (200 μg/ml). (**b**) X-gal and S-gal staining for β-gal: cells were fixed in PBS plus 0.5% glutaraldehyde (5 min) and rinsed in PBS prior to staining. Staining was performed using standard procedures for 2 hours at 37 ^{o}C in PBS plus 1 mg/ml X-gal (Sigma, St. Louis, MO), 1 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$ or with 1.5 mg/ml S-gal (Sigma) and 2.5 mg/ml FAC (see **Figure 3**). (**c**) β-Gal activity assay: The β-gal activity of tumor cells and tissues in mice was measured using the β-gal assay kit (Promega, Madison, WI) with yellow *o*-nitrophenyl β-*D*-galactopyranoside. (**d**) Western blot analysis: Protein was extracted from PC3 tumor cells and was quantified by a protein assay (Bio-Rad, Hercules, CA) based on the Bradford method. Each well was loaded with 30μg protein and separated by 10% SDS-PAGE (Nu-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Primary monoclonal anti-*β*-gal antibody (Promega) and

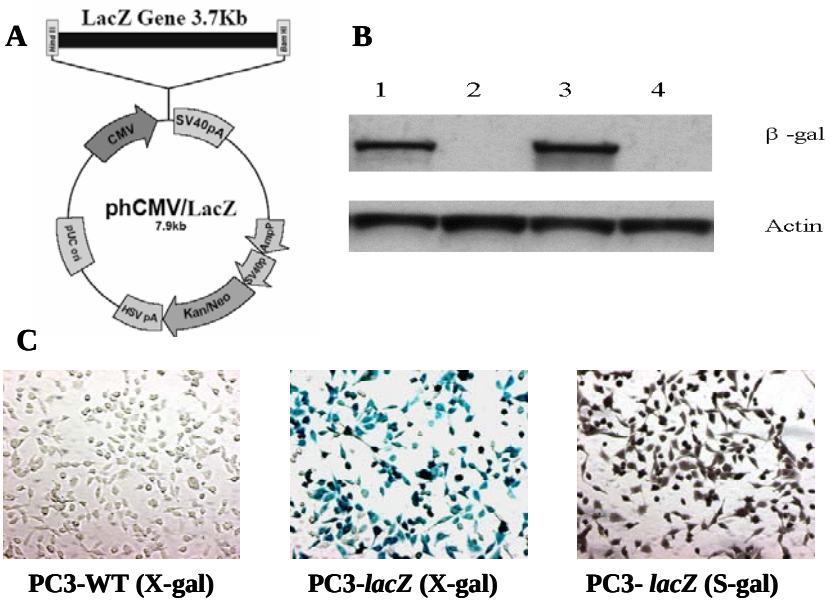

Figure 3 Generation of PC3 cells stably expressing of β-gal. (**A**) Map of recombinant *lacZ* vector (phCMV/*lacZ*). (**B**) Western blot: cell extracts of two transfected lines PC3-*lacZ*1 (lane 1) and PC3-*lacZ* (lane 3), together with PC3-WT (lanes 2 and 4) were examined. (**C**) PC3 wild-type and PC3-*lacZ* cells were stained using X-gal and S-gal: over 90% of PC3-*lacZ* cells were stained blue or black, respectively, while the PC3 wild type cells did not stain.

anti-actin antibody (Sigma) were used as probes at a dilution of 1:5000, and reacting protein was detected using a horseradish peroxidase-conjugated secondary antibody and ECL detection (Amersham, Piscataway, NJ).

(2) *In Vitro* MRI The reporter molecules **M_9** and **M_{10}** (6 μmmol) each in 1:1 DMSO/PBS was added to suspensions of 5×10^6 PC3 wild type and PC3-*lacZ* cells in PBS (1.0 mL) and FAC (3 μmmol) in wells and maintained at 37 ^{o}C. MRI experiments were performed on a 4.7 T Varian Unity INOVA spectrometer. **Figure 5** showed the *in vitro* MR images of **M_9** and **M_{10}** with *lacZ* transfected prostate tumor cells, yielding obvious MRI contrast changes between in WT and *lacZ* transfected PC3 prostate tumor cells, indicating that **M_9** and **M_{10}** both can penetrate prostate tumor PC3 cell membrane and have no apparent cytotoxicity and no physiological perturbation effects on WT and *lacZ* transfected PC3 cells.

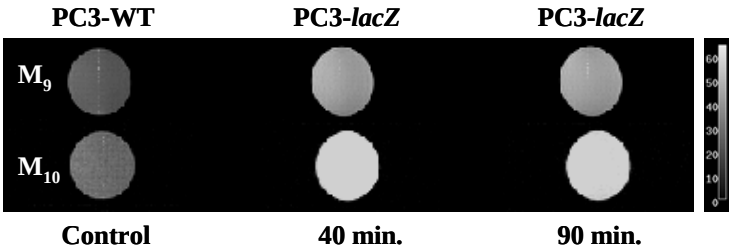

Figure 5 ^{1}H MRI, 200 MHz, TR=300ms, TE=20ms, 1.5mm slice, 128×64, 40×40 mm^2. (**A**) control, **M_9** or **M_{10}** (6 μmol), FAC (3 μmol), 5×10^6 PC3 WT, PBS (0.9 mL), DMSO (0.1 mL); (**B**) **M_9** or **M_{10}** (6 μmol), FAC (3 μmol), 5×10^6 PC3-*lacZ*, PBS (0.9 mL), DMSO (0.1 mL)].

***In Vivo* MRI Studies of M_9 and M_{10}**

(1) Animal model All *in vivo* MRI studies were performed with approval from the Institutional Animal Care and Use Committee (IACUC). Wild type and stably transfected *lacZ* PC3 cells were implanted subcutaneously in the left and right thighs of mice, respectively, when the tumors reached ~0.8 cm in diameter, the mouse was anesthetized (isoflurane/air 2%/98%) and placed into animal coil for imaging. The animal temperature was maintained at 37 ^{o}C by a warm pad with circulating water. Histology analysis confirmed that PC3-*lacZ* tumor section showed over 90% of tissue stained blue for β-gal, while PC3-WT tumor histological section showed little or no blue stain (**Figure 6**). **(2) *In Vivo* MRI with i.v. injection** Mice bearing PC3-WT and PC3-*lacZ* tumors were imaged on a 4.7 T Varian Unity INOVA spectrometer. T1-weighted transaxial images were obtained before and after intravenous injection of the mixture of 0.4 mmol/kg **M_9** and **M_{10}** and FAC. Postcontrast scans were obtained every 5 min for one and half hours. For both reporters **M_9** and **M_{10}**, the MR images of animals showed that there are no time-signal intensity changes between PC3-WT and PC3-*lacZ* tumors before and after **M_9** and

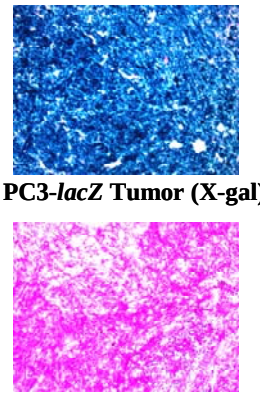

Figure 6

$\mathbf{M_{10}}$ injection (**Figure 7**), indicating that both $\mathbf{M_9}$ and $\mathbf{M_{10}}$ can either be washed out or metabolized very quickly, and can't reach to PC3-WT and PC3-*lacZ* tumors on the thighs with enough amount. Also, we found that mice all died one and half-hours later after intravenous injection of $\mathbf{M_9}$. **(3) *In Vivo* MRI with direct injection into tumors** However, if a solution of $\mathbf{M_{10}}$ (0.4 mmol/kg) and FAC (DMSO/PBS 1:1 V/V') was injected directly into the tumors in a "fan" pattern, strong contrast was detected in the *lacZ* expressing PC3 tumors (**Figure 8**).

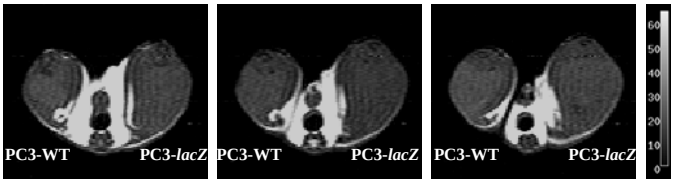

Figure 7 ^{1}H MRI, 200 MHz, TR=250ms, TE=12ms, 1.5mm slice, 128×64, 40×40 mm^2. (**A**) control; (**B**) $\mathbf{M_{10}}$ (0.4 mmol/kg), FAC (0.2 mmol/kg), PBS/DMSO (1:1), i.v. injection].

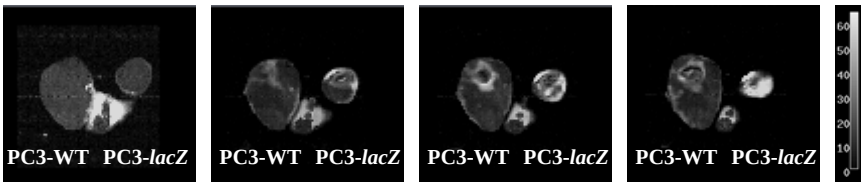

Figure 8 ^{1}H MRI, 200 MHz, TR=250ms, TE=12ms, 1.5mm slice, 128×64, 40×40 mm^2. (**A**) control; (**B**) $\mathbf{M_{10}}$ (0.4 mmol/kg), FAC (0.2 mmol/kg), PBS/DMSO (1:1), i.v. injection].

The further *in vivo* MRI evaluation of $\mathbf{M_9}$ and $\mathbf{M_{10}}$ with *lacZ* transfected prostate tumor is still ongoing. In December 2008, my lab was relocated to a new building, which required remodeling of the new lab and repairs to the refrigeration system and the vacuum instrument. In addition, the Varian unity INOVA 4.7T system was not in service from January to April 2009, and therefore some proposed experiments could not be completed on time. Accordingly, we requested another one-year extension for this project with the remaining funds carried over (no additional cost extension). Based on the *in vitro* and *in vivo* MRI evaluation, we are confident that we will be able to carry out the entire studies proposed in W81XWH-05-1-0593.

RESEARCH ACCOMPLISHMENTS

(1) Finished the *in vitro* evaluation of the reporter molecules $\mathbf{M_9}$ and $\mathbf{M_{10}}$;

(2) Evaluated the reporter molecules $\mathbf{M_9}$ and $\mathbf{M_{10}}$ *in vivo*, respectively, and the results demonstrated this novel mechanism for *in vivo* prostate cancer imaging and evaluation of prostate cancer gene therapy as described in W81XWH-05-1-0593.

REPORTABLE OUTCOMES

Two abstracts have been accepted for presentation on the World Molecular Imaging Congress in Montreal, Canada, Sept. 23-26, 2009.

CONCLUSIONS

Prostate cancer is the most commonly diagnosed cancer and the second most common cause of cancer death in men in the United States. The advent of effective screening measures can sharply decrease the mortality of prostate cancer through detecting this disease at an earlier stage. However, the evidence for mortality benefit from prostate cancer screening has been disappointing to date. Expanding knowledge of prostate cancer biology with combination of imaging technologies would be of considerable value in many ongoing and future clinical prostate cancer diagnosis and gene therapy trials.

Based on the biologic features of prostate cancer, we proposed in this project a new approach for *in vivo lacZ* gene expression assay or *in vivo* prostate cancer imaging. The ultimate objective is to demonstrate the utility and reliability of this new approach to measure β-gal or PSMA activities *in vivo*. We have accomplished a series of target molecules $\mathbf{M_1}$, $\mathbf{M_3}$, $\mathbf{M_5}$, $\mathbf{M_7}$, $\mathbf{M_9}$ and $\mathbf{M_{10}}$, and verified by NMR data. Strong MRI contrast changes of target molecules $\mathbf{M_9}$ and $\mathbf{M_{10}}$ for detection *lacZ in vitro* and *in vivo* demonstrated this novel mechanism described in W81XWH-05-1-0593. We are now focusing on the further *in vivo* studies.

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APPENDICES

(1) Jian-Xin Yu, R. P. Mason, Design and Synthesis of Novel *lacZ* Responsive Enhanced MRI Agent, *The World Molecular Imaging Conference*, #0614, Montreal, Canada, September 23-26, 2009.

(2) Jian-Xin Yu, Dawen Zhao, R. P. Mason, Design and Synthesis of Novel *lacZ* Responsive Enhanced MRI Agent, *The World Molecular Imaging Conference*, #0615, Montreal, Canada, September 23-26, 2009.

DEVELOPMENT OF NOVEL Fe-BASED ^{1}H MRI *lacZ* GENE REPORTERS FOR *IN VIVO* ASSESSMENT

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Presentation: 0614

INTRODUCTION

The *lacZ* gene encoding β-galactosidase (β-gal) has been recognized as the most attractive reporter gene, and its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction. Therefore, its noninvasive *in vivo* detection would be of considerable value in many ongoing and future clinical cancer gene therapy trials.[1] To this end, a variety of *in vivo lacZ* gene reporters has been developed, such as colorimetric,[2] fluorescent,[3-5] bioluminescent,[6] radiotracers for positron emission tomography (PET) or single-photon emission computed tomography (SPECT),[7-9] magnetic resonance imaging (MRI) probes[10,11] and ^{19}F magnetic resonance spectroscopy (MRS) and chemical shift imaging (CSI) reporters.[12-16] We now report the development of a new class of Fe-based ^{1}H MRI *lacZ* gene reporters based on clinically applied iron-depletion agents.

Iron is a critically important metal ion for a wide variety of cellular events. Tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilization of more iron. Many studies indicate that a high level of iron accumulated in animals and humans is associated with both the initiation and the progression of cancers. Cancer cells are also sensitive to iron depletion because of the critical requirement for iron in proteins that play essential roles in DNA synthesis, repair and energy production. Iron depletion as a therapeutic strategy has been applied for cancers. The FDA has already approved five iron chelators for use in anticancer therapy, others are being evaluated in clinical settings.[8,9]

Based on the structural similarities between the iron complexes with the clinically applied iron depletion agents and the known Fe-based ^{1}H MRI contrast agents, we firstly demonstrated that the clinically applied Fe-chelators can act as Fe-based ^{1}H MRI contrast agents producing strong contrast effects (**Table 1**), opening a new era of iron depletion agent as anticancer drug as well as of ^{1}H MRI detection or diagnosis agent.

Chelator	SBH	SIH	SNH
T_1-weighted ^{1}H MRI (Control)			
T_1-weighted ^{1}H MRI (Complex)			

Table 1. ^{1}H-MRI Function of the Fe-Complexes with the clinically applied iron depletion agents: salicylaldehyde benzoyl hydrazone (SBH), salicylaldehyde isonicotinoyl hydrazone (SIH) and its analog salicylaldehyde nicotinoyl hydrazone (SNH). [Conditions: T_1-weighted ^{1}H MRI, 200MHz, TR=300ms, TE=20ms, 1.5mm slice, 128×128, 50-50mm^2. (A) Control, FAC (2.5μmol), PBS (1.5mL); (B) Complex, SBH, SIH or SBH each (5μmol), FAC (2.5μmol), PBS (1.5mL)]

MOLECULAR DESIGN

Prompted by this finding, we now propose a novel molecular design to deliver the in situ formation of Fe-complexes of iron depletion agents specific targeting on cancer by using Gene Directed Enzyme Prodrug Therapy (GDEPT) strategy.

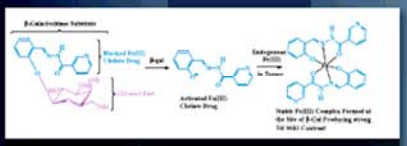


Figure 1. The proposed new ^{1}H MRI approach for *in vivo* detection of *lacZ* gene expression through the in situ formation of Fe-complexes of iron depletion agents.

MOLECULAR DESIGN

Figure 2. The proposed new ^{1}H MRI approach for *in vivo* detection of *lacZ* gene expression through the *in situ* formation of Fe-complexes of iron depletion agents, combining *in vivo* detection and cancer iron chelation therapy.

TARGET MOLECULE SYNTHESIS

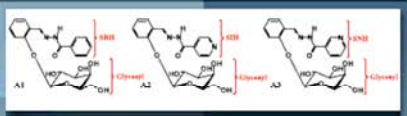
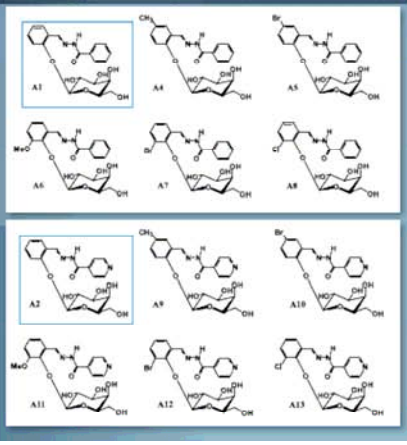
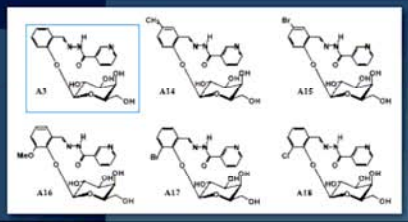


Figure 3. The Structures of the Target Molecules

ALTERNATE MOLECULE SYNTHESIS

Considering the multiple requirements for an ideal *in vivo* reporter, we designed a series of alternate candidates based on the different substituents at different positions, which shall be directly related to the enzyme sensitivity and specificity, aqueous solubility, toxicity, and importantly, the ability of the released aroylhydrazones to complex Fe^{3+} ion (Fe-based ^{1}H MRI effects).





RESULTS

Testing A1–A18 with β-galactosidase G-5160, showed that all, except A7, A8, A12 and A13, can react with β-galactosidase G-5160 in the presence of Fe^{3+}. The released iron depletion agents then spontaneously capture Fe^{3+} readily *in situ* forming stable Fe-complexes, which produce strong Fe-based ^{1}H MRI contrast.

Reporters	A1	A2	A3	A4	A5	A6	A9
T_1-weighted ^{1}H MRI (Control)							
T_1-weighted ^{1}H MRI (Complex)							

Reporters	A10	A11	A14	A15	A16	A17	A18
T_1-weighted ^{1}H MRI (Control)							
T_1-weighted ^{1}H MRI (Complex)							

Table 2. ^{1}H-MRI contrast of the reporters A1–A8, A9–A11 and A14–A18 in the presence of FAC. [Conditions: T_1-weighted ^{1}H MRI, 200MHz, TR=300ms, TE=20ms, 1.5mm slice, 128×128, 50-50mm^2. (A) Control. A1–A6, A9–A11 and A14–A18 each (5μmol), FAC (2.5μmol), PBS (0.1M, pH=4.5, 1.5mL); (B) Complex: A1–A8, A9–A11 and A14–A18 each (5μmol), FAC (2.5μmol), G-5160 (7 units), PBS (0.1M, pH=4.5, 1.5mL)]

CONCLUSIONS

These molecules demonstrate the feasibility of combining anticancer drug iron depletion agents for detection of the *lacZ* reporter gene, opening a new possibility of merging cancer therapy and detection into one approach.

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Development Of Novel *lacZ* Responsive Enhanced Gd-Based MRI Agent

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Presentation: 0615

INTRODUCTION

Gene therapy holds great promise for treating cancer and has been successfully exploited in several clinical trials. A major current obstacle to implementation is to establish a method of assessment of therapeutic gene expression in terms of heterogeneity and longevity in tissues. The *lacZ* gene encoding β-galactosidase (β-gal) has been recognized as the most attractive reporter gene. Therefore, noninvasive detection *in vivo* would be of considerable value in future clinical cancer gene therapy trials.[1] various groups have presented innovative approaches to assessing β-gal activity *in vivo* using gadolinium enhanced contrast ^{1}H MRI or ^{19}F NMR spectroscopy,[2-4] optical[5-13] and radionuclide imaging.[13-16] We now report the exploration of a novel approach to *lacZ* responsive Gd-based ^{1}H MRI agents.

Desreux *et al* demonstrated that, by chelating Gd(phen)HDO3A with Fe(II) to form a highly stable tris-complex, the relaxivity increased 145% at 20MHz and 37°C.[25] Most recently, Tóth *et al* showed that the heterometallic, self-assembled metallostar with six efficiently relaxing GdIII centers, $[Fe\{Gd_2L(H_2O)_2\}_3]^{3+}$, exhibited a particularly high relaxivity for its moderate molecular weight.[26]

Iron is a critically important metal ion for a wide variety of cellular events. Tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilisation of more iron. Many studies indicate that a high level of iron accumulation in animals and humans is associated with both the initiation and the progression of cancers. Cancer cells are also sensitive to iron depletion because of the critical requirement for iron in proteins that play essential roles in DNA synthesis, repair and energy production. Iron depletion as a therapeutic strategy has been applied for cancers. The FDA has already approved five iron chelators for use in anticancer therapy; others are being evaluated in clinical settings.[27]

DESIGN

Prompted by this finding, we now propose a novel class of enzyme activated Gd^{3+}-based MRI contrast agent for *in vivo* detection of β-gal activity combing three features: (A) a signal enhancement group, such as Gd-DTTA; (B) an Fe^{3+} chelating group using the structure of clinical iron chelation therapy drugs; (C) β-*D*-galactose. Following cleavage by β-gal in cells, the released, activated aglycone Fe^{3+}-ligand will spontaneously trap endogenous Fe^{3+} at the site of enzyme activity forming a highly stable complex, exhibiting restricted motion of the Gd^{3+} chelates enhancing relaxivity and providing contrast based on gene (*viz.* enzyme) stimulated local accumulation (Figure 1).

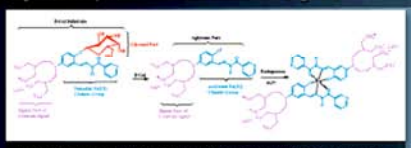
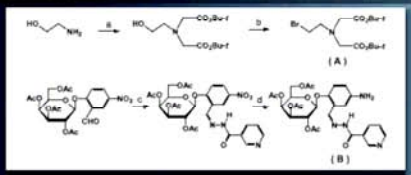


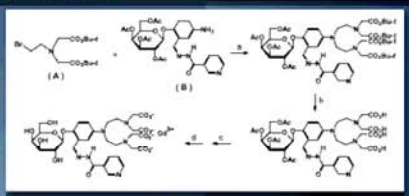
Figure 1. The proposed new mechanism for *in vivo* detection of *lacZ* gene expression through Fe^{3+}-trapped MRI contrast agent formation.

SYNTHESIS



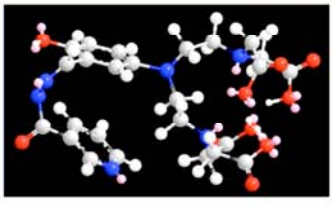
Reaction Conditions: (a) BrCH2CO2But-t, KHCO3, 88%; (b) Ph3P, NBS, 86%; (c) Nicotinic Hydrazide, EtOH, AcOH, Reflux, 85%; (d) H2, Pd/C, 94%.

SYNTHESIS

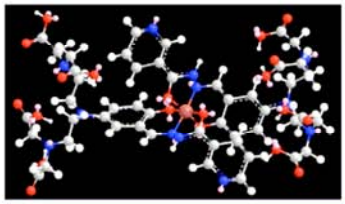


Reaction Conditions: (a) K_2CO_3, MeCN, 38%; (b) CF_3CO_2H, CH_2Cl_2, 52%; (c) $GdCl_3$, Pyridine, 57%; (d) MeOH, MeONa, 75%.

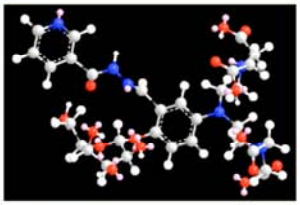
STRUCTURAL FEATURES



Ligand Structure

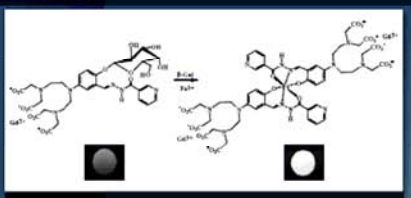


Fe-Complex Structure



Conjugate Structure

RESULTS



Conditions: T1-weighted 1H MRI, 200MHz, TR=300ms, TE=20ms, 1.5mm slice, 128-128, 50-50mm2. (A) Control, sample (5μmol), FAC (2.5μmol), PBS (0.1M, pH=4.5, 1.5mL); (B) Complex, sample (5μmol), FAC (2.5μmol), G-5160 (5 units), PBS (0.1M, pH=4.5, 1.5mL.

CONCLUSIONS

We propose a novel class of enzyme activated Gd^{3+}-based MRI contrast agent for detection of β-gal activity. Synthesis of the substrate has been successful, and the evaluation of this agent with β-gal showed that the released aglycone including the activated Fe^{3+}-ligand and MRI signal enhancement group spontaneously traps Fe^{3+} in the solution forming a highly stable complex, then restricting the motion of the Gd^{3+} chelates enhancing relaxivity. We are now evaluating this agent with β-gal expressing cells and *in vivo*.

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ADDITIONAL INFORMATION

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